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(71) Applicant (*for all designated States except US*): PROMEGA CORPORATION [US/US]; 2800 Woods Hollow Road, Madison, WI 53711-5399 (US).

(72) Inventor; and
(75) Inventor/Applicant (*for US only*): EKENBERG, Steven, J. [US/US]; 1117 Perry Center Road, Mount Horeb, WI 53572 (US).

(74) Agents: FRENCHICK, Grady, J. et al.; Stroud, Stroud, Willink, Thompson & Howard, Suite 300, 25 West Main Street, P.O. Box 2236, Madison, WI 53701-2236 (US).

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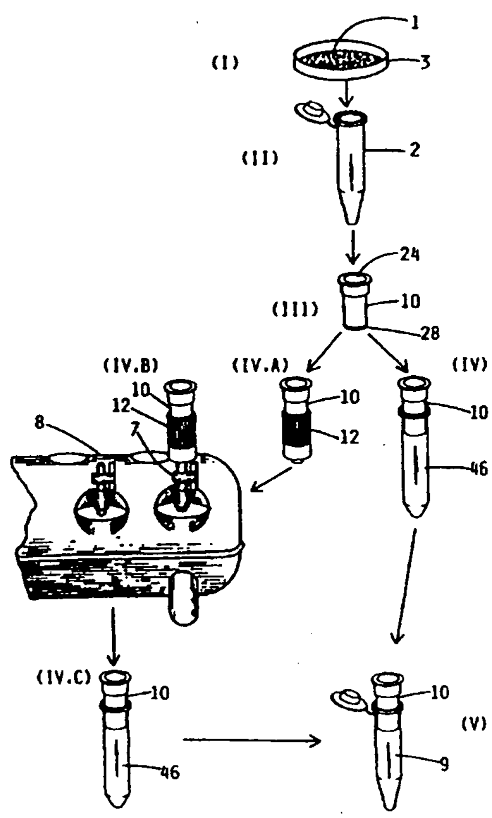
(57) Abstract

A filtration system and method for isolating nucleic acids from cellular debris. The filtration system includes a filter basket (10), including a filter (28) at one end, designed to fit inside both a microcentrifuge tube (9) and a vacuum manifold adapter (12).



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IMPROVED FILTRATION SYSTEM AND METHOD

This application claims the benefit of the priority date under 35 U.S.C. § 119, Provisional United States Application No. 60/026,582, filed 18 September 1996

TECHNICAL FIELD OF THE INVENTION

This invention relates to filtration systems or apparatuses and methods for isolating biological entities. More specifically, and without intending to limit the scope hereof, this invention relates to systems and methods for isolating or separating nucleic acids such as ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) from other material such as cellular debris. The present invention particularly relates to filtration systems or apparatuses and methods for isolating DNA, more particularly for isolating plasmid DNA.

BACKGROUND OF THE INVENTION

It is well known that nucleic acids bind to silica. This property has been used to collect or isolate nucleic acids, particularly DNA, and more particularly plasmid DNA, from cellular debris and from other materials. *See, e.g.,* Patent Cooperation Treaty Published International Patent Application WO 95/06652. Silica materials, in the form of a silica containing resin or in the form of a filter impregnated with silica, are used to isolate or separate nucleic acids contained in living cells from other materials in the cells according to the following general procedure. First, the cells are disrupted or lysed to generate a lysate, i.e., a solution containing the lysed materials. Second, the lysate is treated to remove cellular debris to create a cleared lysate. The cleared lysate is typically created by centrifugation of the lysate to pellet the debris, and by decanting and saving the resulting cleared lysate solution. Third, the cleared lysate solution is contacted with silica, in the presence of a chaotropic agent

designed to promote the binding of the nucleic acid material of interest to the silica. The last step in the process (usually after several intermediate washing or treatment steps) is to release the nucleic acid bound to the silica, e.g., by eluting the nucleic acid from the silica with nuclease-free water.

The silica materials, in the general procedure above, are placed in a filter basket, before or after being bound to nucleic acid materials in a lysate solution. The filter basket is designed to fit into a standard sized microcentrifuge collection tube in such a way that the basket/tube assembly fit inside a standard microcentrifuge. The filter basket is also designed to retain the silica material when loaded with lysate solution, and to permit the lysate solution to pass through the material into the collection tube when subjected to an external force, such as centrifugation or a vacuum. Commercial filter baskets, designed for use in nucleic acid isolation according to the type of procedure outlined above, are available in either of two general configurations. The first such configuration is a filter basket with a substantially flat bottom, designed to be used for spin filtration (i.e., designed to be inserted in a collection tube and spun in a centrifuge) see, e.g. filter baskets disclosed in U.S. patent numbers 5,552,325 and 4,683,058, and the GLASSMAX™ Spin Cartridges (Life Technologies, Inc., Gaithersburg, MD, USA). The other general configuration is a filter basket with a male Ler Lok® fitting protruding from the bottom of the filter basket, a filter basket configuration designed to be used interchangeably in centrifugation or vacuum filtration. See, e.g. QIAprep® Spin Miniprep Kit microcentrifuge spin columns (QIAGEN, Inc. Hilden, Germany)

The two principal commercial filter basket configurations in use today, described briefly above, present difficulties for those wishing to use the baskets to isolate nucleic acids, such as DNA. The flat bottom filter baskets have the advantage of allowing a large volume of solution to be spun through the filter in a single centrifugation step, as the bottom of the filter basket is sufficiently high above the bottom of the collection tube for

a large volume of solution to be coll cted therein while remaining clear of the bottom of the filter basket. However, the flat bottom filter baskets do not permit users to use vacuum filtration to process the contents of the basket, a disadvantage when it comes to the binding and washing steps of the general nucleic acid isolation procedure described above.

The filter baskets with protruding vacuum adapters accord users the flexibility of using centrifugation and/or vacuum filtration to isolate nucleic acids. However, the protruding vacuum adapter portion of the filter basket necessarily limits the volume of solution which can be spun down into the collection tube without contacting the bottom (i.e., the adapter end) of the filter basket. To address this last problem, users of such filter baskets frequently find it necessary to use two or more spin cycles in a microfuge to process the same volume of lysate solution they could have processed in just one spin cycle using a flat bottomed filter basket. Some commercial filter baskets of this last type have a male Luer-Lok® fitting with grooves on the inside surface of part the fitting designed to interfit with a female Luer-Lok® port of a vacuum manifold unit. See, e.g. miniprep microcentrifuge spin columns sold by Qiagen, cited above. The fittings protruding from such filter baskets present the additional problem of solutions spun through the filter collecting in the grooves of the fitting creating potential problems with carry over from one isolation process step to the next.

The present invention is an improved filtration system, one which allows the interchangeable use of centrifugation or vacuum filtration to process volumes of solution comparable to commercial flat bottom filter basket assemblies without the volume limitations and carry-over problems inherent in commercial protruding vacuum adapter filter baskets.

The present invention is also a method of using the improved filtration system of this invention to isolate nucleic acids, such as DNA. The method of this invention is flexible in its application, is especially efficient in the sense that it produces a high yield of isolated nucleic acid, and is less labor

intensive than previously know nucleic acid isolation techniques. The nucleic acid produced in a practice of the method of the present invention is particularly clean and is well suited to such subsequent applications as automated fluorescent DNA sequencing, amplification reactions (especially those using polymerase chain reaction (PCR)), transfection, gene therapy and gene expression. Many other applications of nucleic acid separation or isolated using the present invention will be apparent to one skilled in the art.

Representative, but by no means inclusive prior art includes:

Inventor	Patent Number
Lyman et al.	U.S. 4,683,058
Limb	U.S. 4,832,842
Setcavage et al.	U.S. 5,491,067
Paley	U.S. 4,046,479
Guirguis	U.S. 5,429,803
Diekmann	U.S. 4,956,298
Nieuwkerk	U.S. 5,438,128
Sheer et al.	U.S. 5,124,041

BRIEF SUMMARY OF THE INVENTION

Briefly, in one aspect, the present invention is an improved filtration system. In another aspect the present invention is a method for isolating or separating nucleic acids, particularly DNA, from cellular debris. The system and method of the present invention are designed to be used in conjunction with both a conventional microcentrifuge and any one of a number of different conventional vacuum manifolds.

The filtration system or assembly of the present invention comprises separate filter basket and vacuum adapter components. In an optional practice, the system or assembly also includes a conventional microcentrifuge collection tube.

The vacuum adapter comprises a cylindrical tube, having a first end and a second end. The first end of the vacuum adapter cooperates with the

filter basket to permit it to be inserted therein to restrict its passage though the adapter, and to form a substantially airtight seal therewith. The second end of the adapter is designed to couple with a vacuum source, such as a vacuum manifold. For example, the second end of the vacuum adapter may comprise a female Luer-Lok® coupler. The female Luer-Lok® coupler portion of the vacuum adaptor permits the system to be used with a standard vacuum manifold with male Luer-Lok® ports, such as the Vac-Man® laboratory manifold or the Vac-Man Jr.® laboratory manifold, both commercially available from Promega Corporation, Madison, Wisconsin, U.S.A.

The filter basket and vacuum adapter are designed to cooperate in that the filter basket fits restrictively or frictionally within the first end of the vacuum adapter, forming a substantially air tight seal therewith. Either the outside surface of the filter basket or the inside surface of the vacuum adapter may be slightly flared or tapered, as appropriate, to create the substantially air tight seal. The substantially air tight seal permits a vacuum seal to be formed between the basket filter and adapter when the second end of the adaptor is mounted on a vacuum manifold. In one practice, the filter basket has a high surface area, supported silica filter or filter disc (or a plurality of discs) in one end thereof. It is that silica filter material on which nucleic acid is ultimately isolated in the preferred method of this invention. In this last practice, the end of the filter basket containing the silica is preferably perforated so as to permit fluid to flow therethrough either by centrifugal force or by vacuum assist while retaining the disc or discs therein.

The method of the present invention is a method of purifying or isolating biological material, particularly plasmid DNA, utilizing a choice of method steps used to separate the material of interest from the lysate, a choice made possible by using the filtration system of this invention. Specifically, the method of isolating nucleic acid material of this invention permits the use of either centrifugal force such as that generated in a

microcentrifugation step, or vacuum assist, such as that available by means of a laboratory vacuum manifold. To applicant's knowledge, no other filtration system or method previously disclosed or suggested offers the surprising and unexpected versatility of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a top elevational view of a filter basket of the present invention;

FIG. 2 is a bottom perspective view of a filter basket of the present invention;

FIG. 3 is a bottom view of a filter basket of the present invention;

FIG. 4 is a sectional view of a filter basket of the present invention taken along line 3-3 of FIG. 3;

FIG. 5 is a top elevational view of a vacuum adapter of the present invention;

FIG. 6 is a bottom elevational view of a vacuum adapter of the present invention;

FIG. 7 is a top end-on view of a vacuum adapter of the present invention;

FIG. 8 is a sectional view of a vacuum adapter of the present invention taken along line 7-7 of FIG. 7.

FIG. 9 is a sectional view of filter assembly of this invention comprising a filter basket inserted into a vacuum adapter of the present invention.

FIG. 10 is a sectional view of a filter basket of the present invention contained within a collection tube; and

FIG. 11 is a sectional view of a collection tube.

FIG. 12 is a flow diagram of a method of using a filtration system of this invention to isolate plasmid DNA.

DETAILED DESCRIPTION OF THE INVENTION

The two principal components of the filtration system of this invention, filter basket 10 and vacuum adapter 12 are illustrated in Figures 1-4, and Figures 5-8, respectively. (The same numbers are used in each of the Figures to indicate the same structures.) Filter basket 10 comprises a hollow cylindrical tube 14 having a first end 16 and a second end 18. While the sectional configuration of the filter basket is not critical, in this embodiment, first end 16 comprises a shoulder 20 and a rim 22. Shoulder 20 and rim 22 are configured to cooperate with corresponding structure on the collection tube or capture tube described more completely below and shown in FIG. 11. First end 16 has an open end 24. Second filter basket end 18 has a perforate structure or means i.e., a series of small openings or windows 26. These openings in conjunction with the wall of cylindrical tube 14 define the "basket" structure of the filter basket. It is significant to note at this point that perforate structure or means 26 defines a plane which is generally perpendicular to the axis 27 of filter basket 10. Thus, perforate structure 26 generally defines what could be characterized as a flat bottom at the second end 18 of filter basket 10. This feature of the filter basket is important because the flat bottom permits a larger volume to be left vacant within a cooperating collection tube. The flat bottom of filter basket 10 (as is best shown in FIG. 4), retains at least one silica filter disk within the basket structure. FIG. 4 shows two such silica filter disks 28 retained in the basket structure.

FIGS. 3 and 4 show perforate structure 26 relative to filter means or silica discs 28. Silica discs 28 are restrictively fitted into the bottom of filter basket 10. It is within the contemplation of this invention that other approaches could be used to retain filter discs 28 within hollow cylindrical body 14, e.g., such as ridges, dimples or beads inwardly radially directed structure.

FIGS. 5-8 illustrate a vacuum manifold adapter 12 (sometimes called a filter cup) of the present invention. Manifold adapter 12 has first and

second ends 30 and 32 respectively, defined by a cylindrical body 34. First end 30 defines an opening 36 into which the second end 18 of filter basket 10 is to be inserted. The outside diameter of the second end 18 of filter basket 10 is designed to cooperate with the inside diameter of vacuum adapter 12 so that filter basket 10 restrictively or frictionally fits or is received therewithin to create a substantially airtight seal. (This is best seen in FIG. 9.) Vacuum adapter 12 second end 32 interiorly defines a thread 36. Thread 36 is an internal thread adapted to couple to, e.g., to thread upon, a cooperating structure, such as a Luer-Lok® port of a laboratory vacuum manifold. The Vac-Man® series of laboratory vacuum manifolds constitute a particularly preferred example of such manifolds with which the present invention may be used. The Vac-Man® laboratory manifolds, which have cooperating Luer-Lok® ports are shown in the 1996 Promega Corporation Catalog at page 155, that depiction and disclosure being incorporated by reference herein. Last, second end 32 of the manifold adapter 12 is also interiorly configured to define a discharge opening 38. Materials filtered by means of the present invention flow through discharge opening 38. Vacuum adapter 12 has an optional roughened, i.e., serrated, exterior surface 40 to make gripping, e.g., between the fingers, easier.

FIG. 9 shows in section filter basket 10 inserted into manifold adapter 12. A frictional fit at surface intersection 42 prevents basket 10 from being completely inserted into vacuum adapter 12, and forms a substantially air tight seal therewith. The frictional engagement between filter basket 10 and vacuum adapter 12 defines a chamber 44.

FIG. 10 shows in section a filter basket 10 of this invention inserted within a collection tube 46. Collection tube 46 has an open end 47 and a closed end 49. It is to be noted that no restriction fit is contemplated between filter basket 10 and collection tube 46. Cooperating shoulder 20 and collection tube lip 48 prevent filter basket 10 from sliding completely into collection tube 46. Collection chamber 50 in collection tube 46 is

defined by the walls of the tube and by the flat bottom of filter basket 10. The size of this collection chamber is considerably larger than the chamber formed by commercially available filter baskets with protruding Luer-Lok® couplers and collection tube assemblies. For, the flat bottom of filter basket 10 has no similar protrusions, protrusions which inherently limit the volume of the chamber in the collection tube. The flat bottom of filter basket 10 also avoids the carry over yield problems inherent in the prior art protruding couplers.

FIG. 11 shows a collection tube in section. A collection tube would be used in conjunction with the filter basket of the present invention, when centrifugation is used, e.g. to isolate nucleic acids such as DNA.

It is contemplated that the filter system of the present invention could be used to isolate any one of a number of different substances, including any type of nucleic acid material. However, the most preferred method of using the filter system is to isolate DNA using the filter system of the present invention fit with a filter impregnated with a silica material, most preferably the DNA species isolated is plasmid DNA. A particularly preferred impregnated filter material is the SPEC™ silica disk material commercially available from Ansys Corporation, Irvine, California, USA. Other suitable silica-based materials contemplated for use in the filter baskets used in the present method include the resin material described in PCT Publication Number WO 95/06652, incorporated herein by reference. Any one of a number of different processing techniques can be used to lyse biological material containing a DNA species of interest, and to bind it to the silica component of a filter basket assembly. Suitable materials and methods for doing so are commercially available in kit form from several different sources, including Promega Corporation (Wizard® and Wizard® *Plus* SV DNA Purification Systems) and Qiagen Corporation (QIA® Prep). Suitable techniques for washing and eluting the DNA from the basket filter are also described in the literature.

The following non-limiting example provides a detailed illustration of utilization of one embodiment of the present invention. The specific embodiment of the filter assembly and method of the present invention illustrated in the example are referred to as components of the Wizard® *Plus* SV Minipreps DNA Purification System, a system developed recently at Promega Corporation. The filter basket component of the embodiment of the filtration system of the present invention used in the example is referred to as the Wizard® *Plus* SV Minipreps Spin Column or simply as the Spin Column. The vacuum adapter component is referred to below as a Miniprep Vacuum Adapter. It is to be understood that this example is but one embodiment of the present invention. It is contemplated that additional embodiments of this invention will occur to one skilled in this art in light of the teachings herein.

Example

I. System Overview

Small-scale procedures for isolation of plasmid DNA, better known as "minipreps", are common in molecular biology procedures. See e.g., Ausubel, F.M. *et al.* (1989) *Current protocols in Molecular Biology*, Vol. 2, John Wiley & Sons, NY. Over the years, many miniprep protocols have been developed, but few have proven to be consistently reliable; plasmid DNA yield is often variable in quantity and quality, thus limiting the applications for which it can be used. In addition, known miniprep procedures can be both laborious and time-consuming, particularly when large numbers of minipreps are performed in parallel.

The Wizard® *Plus* SV Minipreps DNA Purification System eliminates many of the problems associated with standard miniprep procedures, by providing a simple and reliable method for rapid isolation of plasmid DNA. This system can be used to isolate any plasmid, but works most efficiently when the plasmid is <20,000bp. When using the standard Wizard® *Plus*

SV Minipreps protocol, the entire minipr p procedure can be completed in 30 minutes or less, depending on the number of samples processed. Furthermore, the plasmid isolated with this protocol can be used directly for automated fluorescent DNA sequencing or restriction enzyme digestion without further manipulation (when high copy number plasmids are used), and also can be used for in vitro transcription reactions when supplemented with a ribonuclease inhibitor such as RNasin® Ribonuclease Inhibitor (Promega Cat.# N2511).

The Wizard® *Plus* SV Minipreps DNA Purification System , as used in this Example, allows one a choice of methods for purification of plasmid DNA. DNA may be purified from the bacterial lysate using microcentrifugation to force the cleared bacterial lysate through the Wizard® *Plus* SV Minipreps Spin Column and to wash the plasmid DNA. Alternatively, the bacterial lysate can be pulled through the Wizard® *Plus* SV Minipreps Spin Column and the plasmid DNA washed using vacuum pressure. Miniprep Vacuum Adapters allow the use of a vacuum manifold (e.g., Vac-Man® Laboratory Vacuum Manifold, 20-sample capacity, Promega Cat.# A7231; or Vac-Man® Jr. Laboratory Vacuum Manifold, 2-sample capacity, Cat.# A7660) to purify plasmid DNA, as described in Section VII.B. The use of a vacuum manifold greatly decreases the amount of time and effort required to purify plasmid DNA and significantly reduces the amount of plastics used compared to previous systems.

Figure 12, a flow diagram, provides an overview of the procedure used to isolate plasmid DNA from bacteria in a culture grown from a single colony, using the Wizard® *Plus* SV Minipreps DNA Purification System. The Wizard® *Plus* SV Minipreps Spin Column is identified as filter basket 10 in Figure 12, to indicate that it is an embodiment of filter basket 10 illustrated in Figures 1-4. Similarly, the vacuum adapter 12 and collection tube 46 are embodim nts of th vacuum adapter 12 and collection tube 46 illustrated in Figures 5-8 and 10-11, respectively. The flow diagram (FIG. 12) illustrates the use of that system to isolate plasmid DNA using either

vacuum filtration, the left path of the diagram, or using centrifugation, the right path of the diagram.

The process illustrated in figure 12 is shown in five steps (I-V). In Step I, a single colony 1 of bacteria is picked from a culture plate 3 and is used to inoculate 1 to 10 ml. of LB medium containing an appropriate antibiotic. The resulting innoculate is incubated overnight (12-16 hours) at 37°C. The overnight culture is centrifuged and the supernatant medium is discarded. The pellet is then thoroughly resuspended in Wizard® *Plus* SV Minipreps Cell Resuspension Solution. In Step II, the resulting solution of resuspended bacteria cells is transferred to a microcentrifuge tube 2. Lysis Solution is then added to the solution of cells, mixed by inversion, and incubated for 1-5 minutes, thereby breaking open the cells and releasing the DNA into solution. Alkaline Protease Solution is added to the resulting lysate, and incubated 5 minutes at room temperature. Neutralization solution is then added, bringing the pH of the solution to neutral or physiological pH. The neutralized solution also contains guanidine, which promotes binding of DNA to silica. The resulting neutralized mixture is then centrifuged to pellet cell debris. In Step III, a in Wizard® *Plus* SV Minipreps Spin Column (shown as spin basket 10 with open end 24 and silica disks 28) is inserted into either a 2 ml collection tube 46 or a Miniprep vacuum adapter 12 on a vacuum manifold 8 depending on whether the vacuum filtration or centrifugation path is to be used to isolate DNA. The supernatant remaining after pelleting the cell debris is decanted into the Wizard® *Plus* SV Minipreps Spin Column.

The vacuum filtration path of Figure 12 (step IVA) shows the Wizard® *Plus* SV Minipreps Spin Column (filter basket 10) inserted and mounted on a port 7 of the vacuum manifold 8. In step IVB of this path, the vacuum is applied to the manifold to remove supernatant from the filter basket 10, followed by vacuum release. The filter basket is washed two times with Wizard® *Plus* SV Minipreps Column Wash Solution, applying vacuum after each wash to remove the wash solution from the basket. In step IV.C, the

filter basket 10 is transf rred to a collection tube 46. The resulting filter basket/tube assembly is then spun in a centrifuge for 2 minutes to remove all remaining Column Wash Solution.

In the alternative, centrifugation, path of Figure 12 (step IV), the Wizard® *Plus* SV Minipreps Spin Column (filter basket 10) is inserted into 2nd collection tube 46, and the supernatant is removed by microcentrifugation. The filter basket 10 is then washed twice with Column Wash Solution using centrifugation to remove the wash solution from the basket. The liquid is discarded after each wash.

Whether centrifugation or vacuum filtration is used to remove the supernatant and to wash the filter basket, the elution step (V) is the same, as indicated by the converging arrows in Figure 12. Specifically, in Step V, Wizard® *Plus* SV Minipreps Spin Column (filter basket 10)is transferred to a sterile 1.5 ml. microcentrifuge tube 9. Nuclease-Free Water is then added, and the resulting assembly is centrifuged to elute plasmid DNA from the filter basket.

II. System Configuration

The Wizard® *Plus* SV Minipreps DNA Purification System used in this Example contained a sufficient amount of reagents and components for 50 isolations from 1-10 ml of culture:

20ml	Wizard® *Plus* SV Cell Resuspension Solution
20ml	Wizard® *Plus* SV Cell Lysis Solution
30ml	Wizard® *Plus* SV Neutralization Solution
20ml	Wizard® *Plus* SV Column Wash Solution
50	Wizard® *Plus* SV Minipreps Spin Columns
50	Collection Tubes (2ml)
550μl	Alkaline Protease Solution
13ml	Nuclease-Free Water

See section X, below for the composition of each of the solutions in this System.

III. Selection and Preparation of Plasmids and *E. coli* Strains

Plasmid DNA was purified from 1-10ml overnight cultures of *E. coli* with the Wizard® *Plus* SV Minipreps DNA Purification System. The yield of plasmid varied depending on a number of factors, including the volume of bacterial culture, plasmid copy number, type of culture medium and the bacterial strain. The protocol used in this Example is for the isolation of plasmid DNA from *E. coli* bacteria.

A. Preparation of *E. coli*

A single, well-isolated colony of *E. coli* was chosen from a fresh Luria-Bertani (LB) agar plate containing antibiotics, and the colony was used to inoculate 1-10ml of LB medium, also containing antibiotics. The use of culture media other than LB is not recommended, as rich culture media, such as Terrific Broth, may lead to very high cell densities that could overload the DNA purification system. The inoculated LB medium was incubated overnight (12-16 hours) at 37°C. The medium was not incubated longer than 16 hours, as cell death and lysis may occur upon longer incubation, resulting in plasmid loss. An optical density reading at 600 Angatroms (OD_{600} reading) of 2-4 indicated that cells had reached the proper growth density for harvesting and plasmid DNA isolation.

Antibiotics were included in all culture media, both agar and liquid, to ensure propagation of only *E. coli* cells containing the plasmid of interest. The plasmid imparts antibiotic resistance to *E. coli*, allowing selection of plasmid-carrying bacteria over those that do not contain the plasmid. *E. coli* progeny that do not receive the plasmid during replication would grow in medium without antibiotics more quickly than those that contain the plasmid. Thus the progeny overgrow the culture of cells containing the plasmid in the absence of the selective pressure of antibiotics. The antibiotic included in the culture media used in this Example was one for which the plasmid to

be isolated contained a resistance gene. Table 1, below, lists several antibiotics commonly used in cloning, as well as preparation of concentrated stock and working solutions and proper storage conditions for maintaining the efficacy of the antibiotic.

Table 1

Antibiotic Preparation and Use in Cloning

Antibiotic	Stock Concentration	Working Concentration	Preparation
Ampicillin (sodium salt)	50mg/ml	50µg/ml	Prepare stock solution in water. Filter-sterilize* and store at -20°C. Add to autoclaved media after cooling to 55°C. Plates containing ampicillin should be stored at 4°C for no longer than 2 weeks.
Note: Carbenicillin may be substituted for ampicillin.	50mg/ml	50µg/ml	Carbenicillin plates can be stored at 4°C for approximately 3 months.
Chloramphenicol	34mg/ml	20-170µg/ml	Dissolve in 100% ethanol for a stock solution and store in aliquots at -20°C for up to one year. Add to autoclaved media after cooling to 55°C. Store plates at 4°C for up to 5 days.
Kanamycin	50mg/ml	30µg/ml	Prepare a stock solution of kanamycin sulfate in water. Filter-sterilize* and store at -20°C. Add to autoclaved media after cooling to 55°C.

Streptomycin	50mg/ml	30µg/ml	Prepare a stock solution of streptomycin sulfate in water. Filter-sterilize* and store at -20°C. Add to autoclaved media after cooling to 55°C.
Tetracycline	12.5mg/ml	10µg/ml for liquid culture, 12.5µg/ml for plates	Prepare a stock solution of tetracycline hydrochloride in 100% ethanol. Store in aliquots at -20°C, protected from light. Add to autoclaved media after cooling to 55°C. Protect media from the light.

*0.2µm filter pore size

B. Plasmid DNA Yield

Plasmid copy number is one of the most important factors affecting plasmid DNA yield. Copy number is determined primarily by the region of DNA surrounding and including the origin of replication in the plasmid. This region, known as the replicon, controls replication of plasmid DNA by bacterial enzyme complexes. Some DNA sequences, when inserted into a particular plasmid, can lower the copy number of the plasmid by interfering with replication. In addition, excessively large DNA inserts can also reduce plasmid copy number. In many cases, the exact copy number of a particular plasmid construct will not be known. However, many of these plasmids will have been derived from a small number of commonly used parent constructs. Table 2 lists some of these plasmids along with reported copy numbers per cell. A theoretical yield based on the published copy number for these plasmids is also presented in Table 2. For the purposes of this Example, high copy number is a

plasmid copy number of >200. Low copy number plasmids have copy numbers <50.

Table 2

Copy Number of Commonly Used Plasmids

Plasmid	Plasmid Size (approximate)	Copy Number	Yield per ml of Culture*	Reference
pGEM®**	2,700bp	300-700	1.8-4.1μg	1
pBluescript®II	2,960bp	300-700	1.8-4.1μg	1
pUC	2,700bp	500-700	2.9-4.1μg	1
pET-3a	4,600bp	>25	~0.23μg	2
pBR322	4,400bp	>25	>0.23μg	3
ColE1	4,500bp	>15	>0.15μg	3
pACYC	4,000bp	10-12	~0.09μg	1
pSC101	9,000bp	≈ 6	~0.12μg	5

*Theoretical plasmid yields were calculated from the reported copy number and size of each plasmid assuming 2.0 x 109 cells per milliliter of culture grown for 16 hours at 37°C.

**U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

References in Table 2:

1. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Laboratory, Cold Spring Harbor, NY, 1.4.
2. Studier, F.W. and Moffat, B.A. (1986) *J. Mol. Biol.* 189: 113.
3. Bolivar, F. *et al.* (1977) *Gene* 2: 95.
4. Kahn, M. *et al.* (1979) *Meth. Enzymol.* 68: 268.
5. Stoker, N.G. *et al.* (1982) *Gene* 18: 335.

C. Bacterial Strain Selection

Endonuclease I is a 12kDa periplasmic protein which degrades double-stranded DNA. This protein is encoded by the gene *endA*. The *E. coli* genotype *endA*1 refers to a mutation in the *endA* gene, which produces an inactive form of endonuclease I. *E. coli* strains with this mutation in the *endA* gene are referred to as *endA* negative (*endA*⁻). Table 3 contains a list of *endA*⁻ *E. coli* strains. The

absence of an *endA*1 in an *E. coli* genotype denotes the presence of the wild type gene which expresses an active endonuclease I. The wild type is indicated as *endA*$^+$. Using the Wizard® *Plus* SV Minipreps DNA Purification System, high quality DNA was easily obtained from both *endA*$^+$ and *endA*$^-$ strains. However, some *endA*$^+$ strains were found to be problematic for a number of applications. Table 4 contains a list of *E. coli* strains that are known to carry the wild type *endA* gene (*endA*$^+$ strains). In general, the use of *endA*$^-$ strains is recommended for use with this system whenever possible, particularly for applications such as automated fluorescent sequencing.

Table 3

endA$^-$ Strains of *E. coli* Recommended for Use with the Wizard® *Plus* SV Minipreps DNA Purification Systems

Strain	Genotype
BJ5183	F^- endA1, sbcBC, recBC, galK, met, thi-1, bioT, hsdR, str^r
DH1	F^-recA1, endA1, gyrA96, thi-1,hsdR17 (r_k^-, m_k^+), supE44, relA1
DH20	DH1, [F' $lacI^q$ lacZ proAB]
DH21	DH1, [F' $lacI^{sq}$ lacZ proAB]
DH5α*	ϕ80dlacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17, r_k^-, m_k^+ supE44, deoR relA1, Δ(lac ZYA-argF), U169.
JM103	$^{endA1.}$ hsdR, sbcB15, hsdR4, thi-1, strA, Δ(lac-pro) [F' traD36 $lacI^q$ZΔM15, proAB]
JM105	thi-1, rpsL, endA1, sbcB15 Δ(lac-proAB), [F' traD36 proAB $lacI^q$ZΔM15]
JM106	F^-, e14$^-$, (mcrA) endA1, gyrA96, thi-1, hsdR17, supE44, relA1, Δ(lac-proAB)
JM107	F^-, e14$^-$ (mcrA), Δ(lac-proAB), thi-1, gyrA96, endA1, hsdR17, relA1, supE44 [F' traD36, proAB, $lacI^q$ZΔM15]
JM108	F^-, e14$^-$, (mcrA), recA1, endA1, gyrA96, thi-1,hsdR17, supE44, relA1, Δ(lac-proAB)
JM109	F^- e14$^-$ (mcrA), recA1, endA1, gyrA96, thi-1,hsdR17, (r_k^-, $m_k^+$$_{\gamma}$ supE44, relA1, D(lac-proAB), [F' traD36, proAB, $lacI^q$ZΔM15]
MM294	F^-endA1, hsdR17, supE44, thi-1
SK1590	F^-, gal, thi-1, sbcBC, endA1, hsdR4
SK1592	F^- thi-1, supE, endA1, sbcBC, hsdR4

SK2267	F^-*endA1, hsdR4, supE44, thi-1, lacZ4 or lac-61, gal-44, ton58,* [*rfa*], *recA1, sbcBC*
SRB	F^- *e14-(mcrA), Δ(mcrCB-hsdSMR-mrr)171, sbcC, recJ, uvrC, umuC::Tn5(kan*^{r}*), supE44, lac, relA1, thi-1, endA1, gyrA96,* [F' *proAB lacI*^{q}*ZΔM15*]
XL1-Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,* [F' *proAB, lacI*^{q}*ZΔM15* Tn*10(tet*^{r}*)*]
XLO	F^-*Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, endA1, thi-1, recA1, gyrA96, relA1, lac,* [F' *proAB, lacI*^{q}*ZΔM15,* Tn*10* (tet^r)] Su^- (nonsuppressing)D

Table 4

endA$^+$ Strains of *E. coli*

Strain	Genotype
BL21(DE3)	F^-, *ompT [lon] hsdS*$_B$ (r_B^- m_B^-; an *E. coli* B strain) with DE3, a lambda prophage carrying the T7 RNA polymerase gene
CJ236	F'*cat*(=pCJ105; M13^{s}Cm^r)*/dut ung1, thi-1, relA1, spoT1, mcrA*
HB101	F^-, *Δ(gpt-proA)62, leuB6, supE44, ara-14, galK2, lacY1, Δ(mcrC-mrr) rpsL20,* (Str^r), *xyl-5 mtl-1, recA13*
JM83	F^-, *ara Δ(lac-proAB), rpsL,* (Str^r), *[φ80*d*lacΔ(lacZ)M15]*
JM101	F'*-traD36 lacI*^{q}*Δ(lacZ)M15, proA*$^+$*B*$^+$*/supE, thi-1, Δ(lac-proAB)*
LE392	F^-, *e14*$^-$*(McrA*$^-$*), hsdR514,* (r_k^-, m_k^+), *supE44, supF58, lacY1 or Δ(lacIZY)6, galK2, galT22, metB1, trpR55*
MC1061	F^-, *araD139, Δ(ara-leu)7696, galE15, galK16, Δ(lac)X74, rpsL,* (Str^r) *hsdR2,* (r_k^-, m_k^+), *mcrA, mcrB1*
NM522 (all NM series strains are *endA*$^+$)	[F' *lacI*^{q}*ZΔM15*], *proA*$^+$*B*$^+$*/supE, thi-1, Δ(lac-proAB) Δ(hsdMS), (mcrB)5* (r_k^- m_k^- *mcr*BC$^-$)
P2392	LE392 *(P2)*
PR700 (all PR series strains are *endA*$^+$)	RR1, *Δ(malB), Δ(argF-lac)U169,* Pro$^+$, *zjc::*Tn5 (Kan^r)
Q358	F^-, *hsdR,* (r_k^-, m_k^+), *supE, fhuA,* (φ80^r)
RR1	HB101, *RecA*$^+$
TB1	JM83, *hsdR,* (r_k^-, m_k^+)
TG1	F' *traD36, lacI*^{q}*Δ(lacZ)M15, proA*$^+$*B*$^+$*/supE, Δ(hsdM-mcrB)5* (r_k^-, m_k^+McrB$^-$), *thi-1, Δ(lac*$^-$*proAB)*
Y1088 (all Y10 series strains are *endA*$^+$)	F^-, *Δ(lacU169), supE, supF, hsdR* (r_k^-, m_k^+) *metB, trpR, fhuA21* [*proC::Tn5*], (pMC9;Tet^{r}Amp^r)

To improve the quality of plasmid DNA isolated from both *endA*$^+$ and *endA*$^-$ strains of *E. coli* Promega's Wizard® *Plus* SV Minipreps DNA Purification Systems include an alkaline protease solution. Alkaline protease, originally identified as subtilisin Carlsberg, is isolated from the bacterium *Bacillus licheniformis*. Guntelberg, A.V. and Otteson, M. (1954) *Compt. Rend. Trav. Lab. Carlsberg* 29, 36. Alkaline protease was added at the end of the lysis step in the present DNA isolation procedure during the preparation of a cleared bacterial lysate, to inactivate endonucleases. The alkaline protease also acts to nonspecifically degrade proteins, thus reducing the overall level of protein contaminants in the cleared bacterial lysate. Aehle, W. *et al.* (1993) *J. Biotechnology* 28, 31; and Van der Osten, C. *et al.* (1993) *J. Biotechnology* 28, 55. Alkaline protease is optimally active at pH 9 or above. When the lysate is neutralized, alkaline protease activity is substantially reduced. U.S. Patent Nos. 5,352,603; and 5,439,817. Section VI further illustrates the use of alkaline protease in the Wizard® *Plus* SV Minipreps DNA Purification System.

IV. Special Considerations for Automated Fluorescent Sequencing

For the isolation of plasmid DNA for use in automated fluorescent sequencing, special consideration should be given to the selection of plasmid type and *E. coli* strain to optimize yield and plasmid quality. Optimal automated fluorescent sequencing results are routinely obtained by using high copy number plasmids and *endA*$^-$ strains of *E. coli*.

Purified plasmid DNA must be within the proper concentration range for successful automated cycle sequencing preferably, not less than 0.1μg/μl, ideally 0.2μg/μl. Kahn, M. *et al.* (1979) *Meth. Enzymol.* 68, 268. To achieve the proper concentration range with plasmid DNA purified using the Wizard® *Plus* SV Minipreps DNA Purification System, plasmid DNA from high copy number plasmids was isolated from bacteria, as described below,

and then concentrated by withdrawing a 1μg aliquot and drying it under vacuum in a Speed-Vac microcentrifuge. The dried DNA was resuspended in 6μl of Nuclease-Free Water and the concentration determined. When working with plasmid DNA from low copy number plasmids, the ethanol precipitation step listed in Section VII.A or B was followed, after the elution step. DNA concentrations were determined by agarose gel/ethidium bromide quantitation prior to any application, an analysis procedure particularly recommended when using low copy number plasmids *Id*. DNA quantitation by spectrophotometric methods, an alternative analysis method, is prone to errors and requires a large amount of sample.

The Wizard® *Plus* SV Minipreps DNA Purification System routinely yielded 3.5-5μg of plasmid DNA when using the pGEM® Vector and DH5α® cells in 1.5ml of LB medium. Larger culture volume were required to obtain sufficient DNA for sequencing lower copy number plasmids. Low copy number plasmid yields varied from 1.5 to 3.0μg of plasmid DNA from 10ml of LB culture medium, when using the pALTER®-1 (*Amp^r*) Stratagene Vector and DH5α® (Life Technologies) cells.

Note: The low copy number of certain plasmids presents special problems for plasmid purification systems because the low ratio of plasmid DNA to contaminating substances within a cell lysate significantly increases the likelihood of copurification of DNA and contaminants by this or any other DNA purification system. Consequently, the use of high copy number plasmids for automated fluorescent sequencing applications is recommended.

V. Additional Materials Used

(Solution compositions are provided in Section X.)

LB agar plates containing antibiotic

LB medium containing antibiotic

Ethanol (95%)

Microcentrifuge capable of 14,000 x g

Sterile 1.5ml microcentrifuge tubes

Optional: ethanol (70%)

Optional: 7.5M ammonium acetate

Optional: Pasteur pipet (either 9" length or with tip extended by heating)

Optional: 10X TE buffer

Optional: tabletop centrifuge capable of 10,000 x g (for harvesting E. coli from 2-10ml of culture medium)

Optional: vacuum manifold (e.g., Vac-Man® or Vac-Man® Jr. Laboratory Vacuum Manifold)

Optional: vacuum source

VI. Production of a Cleared Lysate

The first step in the Wizard® *Plus* SV Minipreps DNA Purification process used herein began with generation of a cleared bacterial lysate. This was accomplished by subjecting the plasmid containing *E. coli* to alkaline lysis (Birnboim, H.C. and Doly, J. (1979) Nucl. Acids Res. 7, 1513), which resulted in a clear, viscous cell lysate. The next step, neutralization of the alkaline lysis, resulted in formation of a white precipitate of cellular debris, with the plasmid DNA solubilized in the supernatant. After centrifugation the liquid was removed and treated to purify the plasmid DNA.

When isolating high copy number plasmids (see Table 2), it is unnecessary to process more than 5ml of bacterial culture to obtain sufficient plasmid DNA for multiple molecular biology applications. If more

than 5ml of culture is processed, the capacity of the Wizard® *Plus* SV Minipreps Spin Column is exceeded and no increase in plasmid yield is observed. When isolating low copy number plasmids, it was necessary to process 10ml of bacterial culture for recovery of sufficient DNA for further applications. However, processing greater than 10ml of culture lead to insufficient clearing of the bacterial lysate, and thus increased contaminants in the plasmid DNA. Therefore, when it became necessary to process greater than 10ml (for low copy number plasmids) or 5ml (for high copy number plasmids), the cultures were divided into 10ml (low copy number) or 5ml (high copy number) aliquots, processed to isolate the DNA, and the eluted DNA was pooled at the end of the purification process.

Alkaline protease was used after addition of the Lysis Solution. This enzyme was added at approximately 250μg per sample to inactivate endonucleases and other proteins that are released during the lysis of the bacterial cells. These proteins can adversely affect the quality of the DNA isolated. Alkaline protease is useful in this procedure because it is optimally active at a pH of 9 or above, the conditions present during the alkaline lysis procedure. Plasmid DNA samples prepared by this method have been tested for carryover of alkaline protease activity. It has been found that less than 10ng of protease remains in the DNA sample (<0.004% of the protease added) and that the protease is substantially less active below pH 9. The DNA prepared by this procedure was tested extensively in a range of molecular biology applications including fluorescent sequencing, restriction enzyme digestion, Promega's TNT® Coupled Transcription/Translation System and cloning. The results indicate that protease carryover does not affect these applications. However, if alkaline protease activity is a concern, the enzyme can be irreversibly inactivated by heating the DNA sample for 5 minutes at 65°C.

Below is a detailed summary of the steps followed to produce a cleared lysate from a bacteria culture, to begin an isolating procedure using

the Wizard® *Plus* SV Minipreps DNA Purification System. Prior to beginning the procedure, the Wizard® *Plus* SV Column Wash Solution was diluted by adding 35ml of 95% ethanol for a final volume of 55ml:

1. 1-5ml of bacterial culture containing high copy number plasmid or 10ml of bacterial culture containing low copy number plasmid was pelleted by centrifugation for 5 minutes at 10,000 x g using a tabletop centrifuge. The supernatant was poured off, and the tube blotted upside down on a paper towel to remove excess liquid.

2. 250μl of Wizard® *Plus* SV Cell Resuspension Solution was added and completely the cell pellet resuspend by vortexing well or pipetting. It is essential to thoroughly resuspend the cells. If not already in a microcentrifuge tube, the resuspended cells were transferred to sterile 1.5ml microcentrifuge tube(s).

   Note: To prevent shearing of the chromosomal DNA and thus contamination of plasmid DNA, it is recommended one not vortex after this step. One should only mix by inverting the tube(s).

3. 250μl of Wizard® *Plus* SV Cell Lysis Solution was then added and mixed by inverting the tube four times (not vortexed). The resulting solution was incubated until the cell suspension cleared, approximately 1-5 minutes.

   Note: It is important to observe partial clearing of the lysate before proceeding to addition of the Alkaline Protease Solution (Step 4). However, do not incubate longer than 5 minutes.

4. 10μl of Alkaline Protease Solution was added and mixed by inverting the tube four times. The resulting mixture was then incubated for 5 minutes at room temperature.

   Note: Incubation for more than 5 minutes may result in nicking of the plasmid DNA.

5. 350μl of Wizard® *Plus* SV Neutralization Solution was added and immediately mixed by inverting the tube four times.

6. The bacterial lysate was centrifuged at 14,000 x g in a microcentrifuge for 10 minutes at room temperature.

VII. Plasmid DNA Purification

The Wizard® *Plus* SV Minipreps DNA Purification System allows a choice of methods for purification of plasmid DNA (See e.g. the two isolation paths illustrated in FIG. 12). Specifically, plasmid DNA may be purified from the bacterial lysate using microcentrifugation to force the cleared bacterial lysate through the Wizard® *Plus* SV Minipreps Spin Column and to wash the plasmid DNA. Alternatively, a vacuum can be used to pull the bacterial lysate through the Spin Column and to wash the plasmid DNA. Miniprep Vacuum Adapters allow the use of a vacuum manifold (e.g., Vac-Man® or Vac-Man® Jr. Laboratory Vacuum Manifold) and vacuum source for DNA purification. The Vac-Man® Jr. Laboratory Vacuum Manifold is a #8 neoprene stopper with two, one-way Luer-Lok® stopcocks as a single unit that easily inserts into a 1- or 2-liter sidearm flask. It is used in conjunction with a water aspirator-type vacuum source. The Vac-Man® Laboratory Vacuum Manifold is a single, self-contained unit that accommodates up to 20 samples.

To use the Wizard® *Plus* SV Minipreps Spin Column, label it with the corresponding sample number and insert it into the Miniprep Vacuum Adapter which has been attached to the vacuum manifold. The Miniprep Vacuum Adapter/Spin Column junction is designed so that the Spin Column does not touch the bottom of the Miniprep Vacuum Adapter. This allows for wear of the Miniprep Vacuum Adapter while retaining a vacuum seal. The cleared bacterial lysate (Section VI) is added to the Spin Column. A vacuum is applied which pulls the solution through the Spin Column leaving the plasmid DNA on the Column. Wizard® *Plus* SV Column Wash Solution

is then applied to th Spin Column and pulled through by vacuum. The Spin Column is removed from the vacuum manifold prior to eluting the DNA. Elution of DNA is accomplished by centrifugation of the Spin Column after adding Nuclease-Free Water (Section VII.A or B).

Section VII.A of this Example describes plasmid DNA purification via centrifugation, while Section VII.B details plasmid DNA purification via vacuum.

A. By Centrifugation

Plasmid DNA purification filtration units were prepared by inserting one Wizard® *Plus* SV Minipreps Spin Column into one 2ml Collection Tube for each sample. The resulting filtration units are then used to isolate plasmid DNA as follows.

1. Cleared lysate prepared as described in VI, above was transferred (Section VI Step 6; approximately 850μl) to the Wizard® *Plus* SV Minipreps Spin Column, by decanting. The transference was done carefully to avoid disturbing or transferring any of the white precipitate with the supernatant. If the white precipitate was accidentally transferred to the Spin Column, the Spin Column contents were poured back into a sterile 1.5ml microcentrifuge tube and centrifuged for another 5-10 minutes at 14,000 x g prior to decanting again. The resulting supernatant was transferred into the same Spin Column used initially for this sample. The Spin Column was reused in later steps below, but only for the same sample.

2. The supernatant was centrifuged at 14,000 x g in a microcentrifuge for 1 minute at room temperature. The Wizard® *Plus* SV Minipreps Spin Column was then removed from the tube and the flowthrough from the Collection Tube was discarded. The Spin Column was then reinserted into the Collection Tube.

3. 750μl of Wizard® *Plus* SV Minipreps Column Wash Solution was added to the Spin Column.

4. The resulting Spin Column collection tube assembly was centrifuged at 14,000 x g in a microcentrifuge for 1 minute at room temperature.

5. The Wizard® *Plus* SV Minipreps Spin Column was removed from the collection tube and the flowthrough discarded. The Spin Column was reinserted into the Collection Tube.

6. 250μl of Wizard® *Plus* SV Minipreps Column Wash Solution was added to the Spin Column.

7. The resulting Spin Column collection tube assembly was centrifuged at 14,000 x g in a microcentrifuge for 2 minutes at room temperature.

8. The Wizard® *Plus* SV Minipreps Spin Column was transferred to a new, sterile 1.5ml microcentrifuge collection tube, being careful not to transfer any of the Wizard® *Plus* SV Column Wash Solution with the Spin Column. If the Spin Column had Column Wash Solution associated with it, the assembly was centrifuged again for 1 minute at 14,000 x g before being transferred to the new 1.5ml collection tube.

9. The plasmid DNA was eluted by adding 100μl of Nuclease-Free Water to the Wizard® *Plus* SV Minipreps Spin Column. The Column collection tube assembly was then centrifuged at 14,000 x g for 1 minute at room temperature in a microcentrifuge.

10. After eluting the DNA, the assembly was removed from the 1.5ml microcentrifuge collection tube and the Wizard® *Plus* SV Minipreps Spin Column was discarded. If using a low copy number plasmid with fluorescent sequencing it was found essential to follow the note below.

Note: To ensure optimal automated fluorescent sequencing results when using low copy number plasmids and high culture volumes, it is essential that an ethanol precipitation step be performed after eluting the DNA from the Spin Column to concentrate the DNA. This precipitation step should be performed using the following reagents and conditions:

11. The eluted DNA was concentrated as follows: 50μl of 7.5M ammonium acetate and 375μl of 95% ethanol were added to a 100μl sample of eluted DNA to precipitate DNA. The resulting mixture was centrifuged at 14,000 x g for 15 minutes at room temperature. The supernatant was carefully aspirated using a 9" Pasteur pipet, or a regular Pasteur pipet with the tip extended by heating, to avoid disturbing the translucent DNA pellet. The pellet was rinsed briefly in 250μl of 70% ethanol, and then centrifuged at 14,000 x g for 5 minutes. The ethanol was then aspirated, the pellet air dried for 3-5 minutes to allow the residual ethanol to evaporate. The dried DNA pellet was resuspended in 10-25μl of Nuclease-Free Water; the volume of water used to resuspend the DNA pellet was necessarily determined empirically, depending on the concentration of the DNA. Agarose gel/ethidium bromide was used to quantify the DNA isolated according to this procedure.

12. Finally, the concentrated, isolated DNA was prepared for storage in TE buffer, by adding 10μl of 10X TE buffer to the 100μl of eluted DNA. The microcentrifuge tube of DNA in TE buffer was stored at —20°C or below.

Note: DNA is stable in water without addition of a buffer, such as TE buffer, if stored at —20°C or below. DNA is stable at 4°C in TE buffer.

It is important to consider the consequences of DNA storage in TE buffer. Specifically the EDTA in TE buffer may inhibit the

activity of some enzymes by chelating magnesium which is required as a cofactor for enzyme activity. Addition of TE buffer (and the presence of EDTA) will be most problematic when DNA concentrations are low and larger volumes of the DNA solution must be added to a reaction. TE buffer cannot be used in fluorescent DNA sequencing.

B. By Vacuum

One Miniprep Vacuum Adapter with Luer-Lok® fitting was attached to one port of a vacuum manifold (e.g., Promega's Vac-Man® or Vac-Man® Jr. Laboratory Vacuum Manifold). A Wizard® *Plus* SV Minipreps Spin Column was inserted into the Miniprep Vacuum Adapter until snugly in place.

1. The cleared bacterial lysate from Section VI Step 6 was transferred into the Wizard® *Plus* SV Minipreps Spin Column by decanting the liquid. If any of the precipitate was transferred to the Spin Column, the liquid and precipitate were poured back into microcentrifuge tube and centrifuged again for 1 minute at 14,000 x g (see Step VI.6), before only the liquid was decanted into the Spin Column. The Spin Column was reused, but only for the same sample.

2. A vacuum was applied to pull the liquid through the Wizard® *Plus* SV Minipreps Spin Column. When all liquid had been pulled through the column, the vacuum was released.

3. 750μl of the Wizard® *Plus* SV Column Wash Solution previously diluted with 95% ethanol, was added to the Spin Column (see Section VI).

4. A vacuum was then applied to pull the Wizard® *Plus* SV Column Wash Solution through the Wizard® *Plus* SV Minipreps Spin Column.

When all the liquid had been pulled through the Spin Column, the vacuum was released.

5. The wash procedure was repeated, using 250μl of Wizard® *Plus* SV Column Wash Solution. A vacuum was applied to pull the liquid through the Wizard® *Plus* SV Minipreps Spin Column.

6. When all the Wizard® *Plus* SV Column Wash Solution had been removed by vacuum, the vacuum was turned off and the Wizard® *Plus* SV Minipreps Spin Column transferred to a 2ml Collection Tube. The resulting colum and collection tube assembly was centrifuged at 14,000 x g for 2 minutes to remove any residual Column Wash Solution. The 2ml Collection Tube and any liquid collected during this step were discarded after centrifugation.

7. The Wizard® *Plus* SV Minipreps Spin Column was transferred to a new, sterile 1.5ml microcentrifuge tube.

8. The plasmid DNA was eluted by adding 100μl of Nuclease-Free Water to the Wizard® *Plus* SV Minipreps Spin Column, and by centrifuging the spin column and water at 14,000 x g for 1 minute at room temperature in a microcentrifuge tube.

9. After eluting the DNA, the Wizard® *Plus* SV Minipreps Spin Column was removed from the 1.5ml microcentrifuge tube and discarded. If using a low copy number plasmid, particularly with automated fluorescent sequencing, it is essential to follow the note below.

Note: To ensure optimal automated fluorescent sequencing results when using low copy number plasmids and high culture volumes, it is essential that an ethanol precipitation step be performed after eluting the DNA from the Wizard® *Plus* SV Minipreps Spin Column, to concentrate the DNA. This precipitation step should be performed using the following reagents and conditions:

10. The eluted DNA was concentrated as follows: 50μl of 7.5M ammonium acetate and 375μl of 95% ethanol were added to a 100μl sample of eluted DNA. The resulting mixture was centrifuged at 14,000 x g for 15 minutes at room temperature. The supernatant was carefully aspirated using a 9" Pasteur pipet, or a regular Pasteur pipet with the tip extended by heating, to avoid disturbing the translucent DNA pellet. The pellet was rinsed briefly in 250μl of 70% ethanol, and then centrifuged at 14,000 x g for 5 minutes. The ethanol was then aspirated, the pellet air dried for 3-5 minutes to allow the residual ethanol to evaporate. The dried DNA pellet was resuspended in 10-25μl of Nuclease-Free Water; the volume of water used to resuspend the DNA pellet was necessarily determined empirically, depending on the concentration of the DNA. Agarose gel/ethidium bromide was used to quantify the DNA isolated according to this procedure.

12. Finally, the concentrated, isolated DNA was prepared for storage in TE buffer, by adding 10μl of 10X TE buffer to the 100μl of eluted DNA. The microcentrifuge tube of DNA in TE buffer was stored at —20°C or below.

Note: DNA is stable in water without addition of a buffer, such as TE buffer, if stored at —20°C or below. DNA is stable at 4°C in TE buffer.

It is important to consider the consequences of DNA storage in TE buffer. Specifically the EDTA in TE buffer may inhibit the activity of some enzymes by chelating magnesium which is required as a cofactor for enzyme activity. Addition of TE buffer (and the presence of EDTA) will be most problematic when DNA concentrations are low and larger volumes of the DNA solution must be added to a reaction. TE buffer cannot be used in fluorescent DNA sequencing.

VIII. Additional Considerations

A. Performing Large-Scale Plasmid Preps

For larger-scale plasmid preps, Wizard® *Plus* Midipreps (10-100ml cultures; Cat.# A7640), Maxipreps (100-500ml cultures; Cat.# A7270) or Megapreps (500-1,000ml cultures; Cat.# A7300) DNA Purification Systems can be used. These systems are designed for plasmid purifications in quantities up to 500μg, 1mg and 2mg respectively, for high copy number plasmids.

B. Purification of Plasmid DNA from Low Melting/Gelling Temperature Agarose Gels

The Wizard® *Plus* SV Minipreps DNA Purification System is not designed for use with plasmid DNA in agarose. To purify plasmid DNA (circular or linearized) from low melting/gelling temperature agarose band slices, we recommend Promega's Wizard® PCR Preps DNA Purification System (Cat.# A7170) or AgarACE® Enzyme (Cat.# M1741).

IX. Troubleshooting

Symptom	Possible Cause	Comments
Poor cell lysis	Too many bacterial cells in culture medium	Use only LB medium to grow bacteria. All media should contain antibiotics. Use only recommended culture volumes for low and high copy number plasmids (10ml maximum for low copy and 5ml for high copy number plasmids).

	Poor resuspension of bacterial cell pellet	Cell pellet, during preparation of a cleared lysate, must be thoroughly resuspended prior to cell lysis. Vortex or pipet pellet with Wizard® *Plus* SV Cell Resuspension Solution. No cell clumps should be visible after resuspension.
No plasmid DNA purified	Ethanol not added to the Column Wash Solution	Prepare the Wizard® *Plus* SV Column Wash Solution as instructed in Section VI before beginning the procedure.
	Inaccurate quantitation of plasmid DNA yield	Quantitate plasmid DNA yield via agarose gel/ethidium bromide electrophoresis.
	DNA floats out of well during loading of gel for quantitation, due to ethanol remaining in purified DNA	After elution and precipitation of plasmid DNA, dry any remaining ethanol from the DNA pellet before adding water to resuspend.
	Improper storage of DNA	Store DNA at -20°C or below in water or in TE buffer at 4°C to -70°C. Quantitate DNA prior to storage.
	Bacterial culture too old	Inoculate antibiotic containing media with freshly isolated bacterial colony from an overnight plate. Incubate at 37°C for 12-16 hours with shaking.

	Low copy number plasmid used	Know the copy number of plasmid used and follow specialized instructions for use of low copy number plasmids. Culture volume should not exceed 5ml for isolation of high copy number plasmids and 10ml for low copy number plasmids.
	Plasmid DNA yield was not accurately quantitated	Use agarose gel/ethidium bromide quantitation; do not rely on the accuracy of spectrophotometric quantitation.
Nicking of plasmid DNA	Over-incubation during the alkaline lysis step	Total incubation of cell suspension with the Lysis Solution and Alkaline Protease should not exceed 5 minutes.
No results with automated fluorescent sequencing	Too little DNA was added to the sequencing reaction	Inoculate fresh LB medium with a newly isolated *E. coli* colony. Purify plasmid DNA and quantitate with agarose gel/ethidium bromide electro-phoresis.
	TE buffer was used for DNA elution	Repurify plasmid DNA and elute in Nuclease-Free Water.
	Low copy number plasmid used	Follow directions for precipitation of plasmid DNA after eluting when using low copy number plasmids.
	Plasmid concentration not accurately quantitated	Ethidium bromide gel electrophoresis must be used to accurately quantitate plasmid DNA.
No restriction digestion	Concentration of restriction enzyme, length of digestion need to be increased	Increase the amount of restriction enzyme and/or the length of incubation time. Digest at suggested temperature and in the optimal buffer for the restriction enzyme used.

Genomic DNA contamination	Vortexing or overmixing resulted in genomic DNA contamination	Do not vortex samples after addition of Lysis Solution to prevent shearing of genomic DNA.
	Wrong reagents used	Make certain that Column Wash Solution is diluted with ethanol before use. Note that Wizard® *Plus* and Wizard® *Plus* SV components are not interchangeable.
DNA yields on gel look low compared to spectro-photometric readings	Traces of contaminants may be present in the eluted DNA which inflate the spectrophotometer readings	Phenol:chloroform extract and precipitate DNA then wash with 70% ethanol before repeating spectrophotometric readings. Alternatively, quantitate DNA by agarose gel/ethidium bromide electrophoresis for superior quantitation results.

X. Buffers and Solutions

Wizard® *Plus* SV Cell Resuspension Solution

50mM	Tris-HCl, pH 7.5
10mM	EDTA
100μg/ml	RNase A

Wizard® *Plus* SV Cell Lysis Solution

0.2M	NaOH
1%	SDS

Wizard® *Plus* SV Neutralization Solution

4.09M	guanidine hydrochloride
0.759M	potassium acetate
2.12M	glacial acetic acid

Final pH is approximately 4.2.

10X TE buffer

100mM	Tris-HCl, pH 7.5
10mM	EDTA

Wizard® *Plus* SV Column Wash Solution

162.8mM	potassium acetate
27.1mM	Tris-HCl, pH 7.5

LB medium

10g	casein peptone
5g	yeast extract
5g	NaCl
15g	agar (for plates only)

Dissolve in 1L of distilled water. Autoclave and cool to 55°C before adding antibiotic (see Table 1).

CLAIMS:

1. A filter assembly for filtering materials by means of centrifugal force or vacuum assist comprising:

a filter basket comprising a cylindrical tube, the filter basket having a first end and a second end, the first end of the filter basket being open and the second end of the filter basket being adapted to receive and hold a filtration means, the adaptation comprising a perforate structure at the filter basket second end, the perforate structure being sufficiently perforate to support and retain said filter means while permitting fluid to flow therethrough; and

a vacuum manifold adapter comprising a cylindrical vacuum adapter having a first end and a second end, the first end of the adapter being interiorly configured frictionally to receive the second end of the filter basket, and the second end of the adapter being interiorly configured to couple to a vacuum manifold and to define a discharge opening.

2. A filter assembly according to claim 1, wherein the perforate structure defines a flat bottom at the second end of the filter basket.

3. A filter assembly according to claim 1, wherein the filter means comprises silica.

4. A filter assembly according to claim 1, wherein the second end of the filter basket is further adapted to fit into a collection tube.

5. A filter assembly according to claim 4, further comprising the collection tube.

6. A method for isolating a nucleic acid material using a filter assembly for filtering materials by means of centrifugal force or vacuum assist comprising:

a filter basket comprising a cylindrical tube, the filter basket having a first end and a second end, the first end of the filter basket being open and the second end of the filter basket being adapted to receive and hold a filtration means, the adaptation comprising a perforate structure at the filter basket second end, the perforate structure being sufficiently perforate to support and retain said filter means while permitting fluid to flow therethrough; and

filter means fitted into the filter basket, the filter means being adapted to reversibly bind the nucleic acid material;

a vacuum manifold adapter comprising a cylindrical vacuum adapter having a first end and a second end, the first end of the adapter being interiorly configured frictionally to receive the second end of the filter basket, and the second end of the adapter being interiorly configured to couple to a vacuum manifold; and

a collection tube having an open end and a closed end, the end of the filter basket adjacent to the filter means fitting into the open end of the collection tube;

the method comprising the steps of:

a. providing cells containing the nucleic acid material;

b. lysing or disrupting the cells, thereby forming a lysate;

c. transferring the lysate solution to the filter basket, in the presence of a chaotropic agent capable of promoting binding of the nucleic acid material to the silica;

d. removing the lysate solution from the filter basket using removal steps comprising either (i) placing the filter basket in the collection tube and subjecting it to centrifugal force, or

(ii) fitting the filter basket onto the first end of the manifold adapter and the second end onto a vacuum manifold and subjecting it to a vacuum;

e. washing the filter basket with a wash solution, using centrifugation or a vacuum; and

f. eluting the nucleic acid material from the filter basket by adding an elution buffer or water to the basket, placing the basket in a second collection tube and subjecting it to centrifugal force.

7. A method according to claim 6, wherein the nucleic acid material is isolated using the filter basket fitted with the filter means comprising silica.

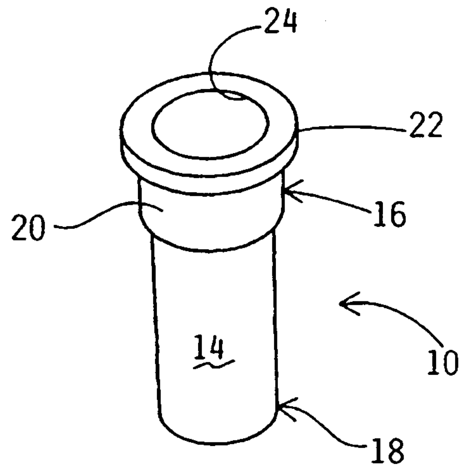

FIG. 1

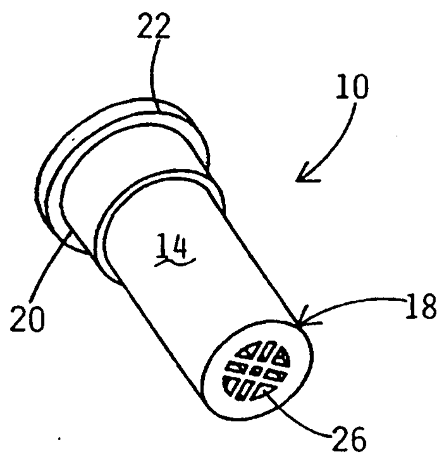

FIG. 2

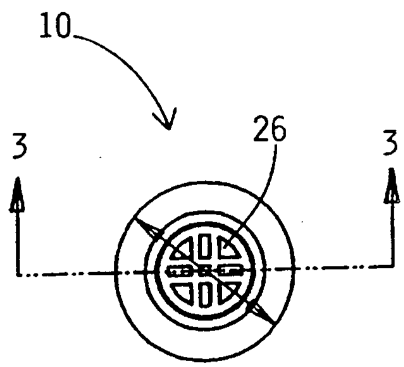

FIG. 3

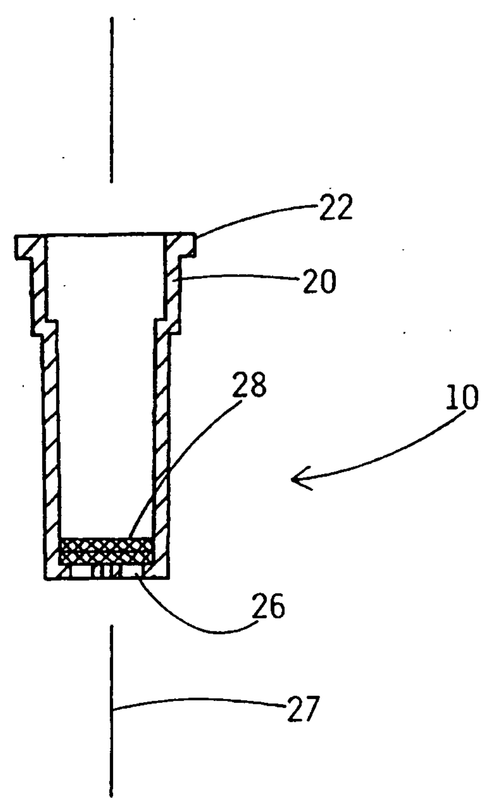

FIG. 4

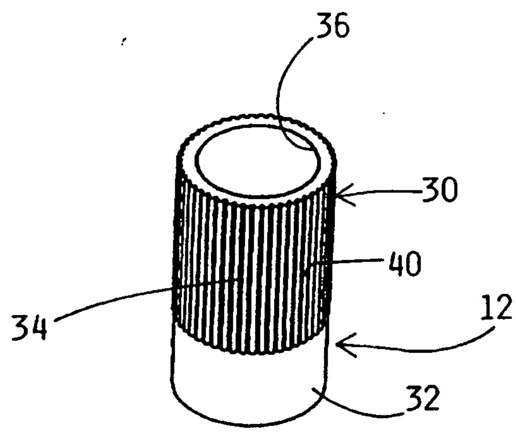

FIG. 5

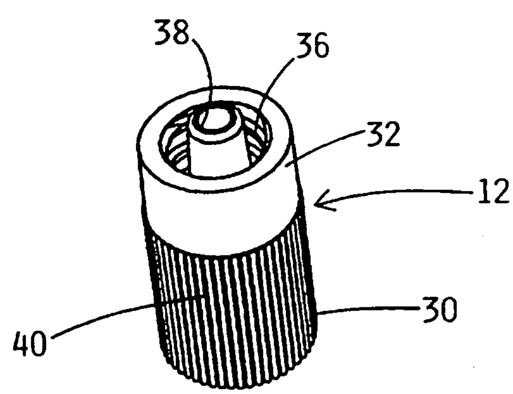

FIG. 6

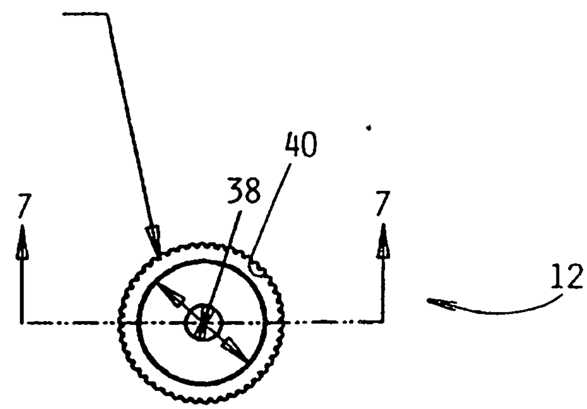

FIG. 7

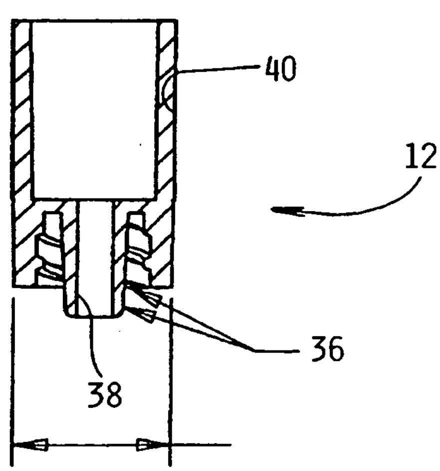

FIG. 8

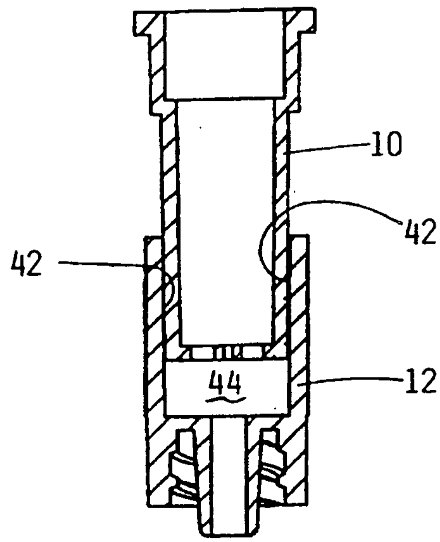

FIG. 9

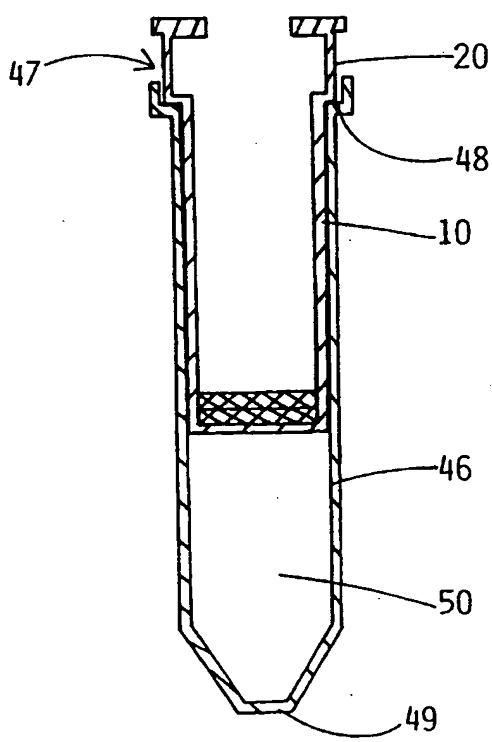

FIG. 10

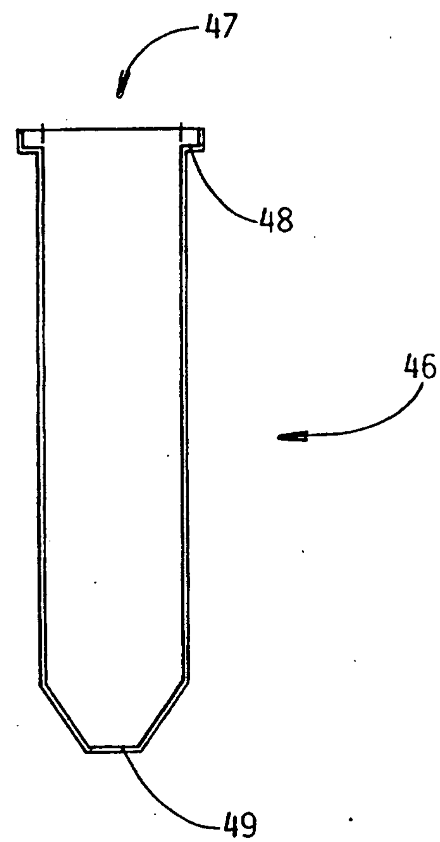

FIG. 11

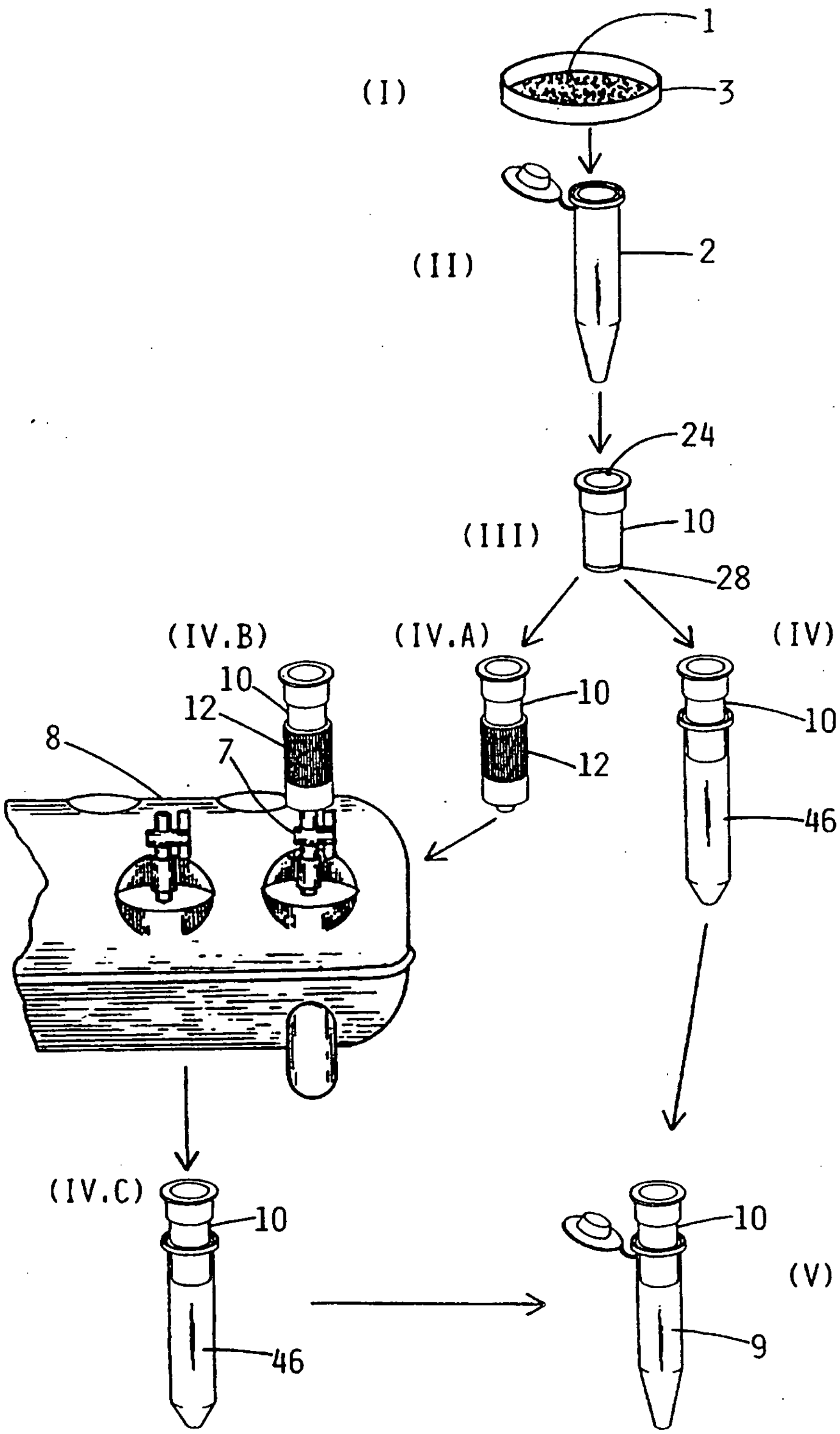

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/16634

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :B01L 11/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/72, 101, 102; 436/177; 210/406, 455; 494/16; 435/6, 91.1, 270, 287.2, 288.1, 304.1; 935/77

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS search terms: filter?, centrifug?, vacuum or evacuat? or suction?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US, A, 4,675,110, (FAY) 23 June 1987 (23.06.87), see figure 1.	1-2, 4-5 ---------- 3

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

04 DECEMBER 1997

Date of mailing of the international search report

02 FEB 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
JAN M. LUDLOW
Telephone No. (703) 308-0651

Form PCT/ISA/210 (second sheet)(July 1992)*

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

422/72, 101, 102; 436/177; 210/406, 455; 494/16; 435/6, 91.1, 270, 287.2, 288.1, 304.1; 935/77

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